FOODBORNE PATHOGENS AND DISEASE
Volume 6, Number 7, 2009

DOI: 10.1089/fpd.2009.0296

Original Article

Ecology of *Enterococcus faecalis* and Niche-Adapted or Non-Niche-Adapted *Enterococcus faecium* in Continuous-Flow Anaerobic Cultures

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Abstract

An anaerobic continuous-flow culture of chicken gastrointestinal microflora (CCF) and pure cultures of *Enterococcus faecalis* I.2 were used for survivability studies of niche-adapted and non-niche-adapted *Enterococcus faecium* isolates. CCF eliminated non-CCF niche-adapted glycopeptide-resistant *E. faecium* 47 (GRE47) at a rate of 1.01 $\log_{10}$ cfu/mL/day, whereas CCF niche-adapted *E. faecium* I.3^{rif} survived in CCF at 4.5–6.5 $\log_{10}$ cfu/mL. In continuous-flow monocultures of GRE47 (8.93 $\log_{10}$ cfu/mL), the addition of 100 mL (9.5% total volume) of CCF resulted in the displacement of GRE47 in 14 days at a rate of 0.66 $\log_{10}$ cfu/mL/day. Pure continuous-flow cocultures were used to assess a direct inhibitory effect of *E. faecalis* I.2 on *E. faecium* isolates. In cocultures of *E. faecalis* I.2 and GRE47, GRE47 was eliminated from the culture at a rate of 1.24 $\log_{10}$ cfu/mL/day. In cocultures of *E. faecalis* I.2 and *E. faecium* I.3^{rif}, the *E. faecium* I.3^{rif} population fluctuated, but was 6.86 $\log_{10}$ CFU/mL on day 21. A fit subset of the *E. faecium* I.3^{rif} population survived in CCF and with *E. faecalis* I.2 alone. No subset of the non-niche-adapted *E. faecium* GRE47 was able to survive under the same conditions. The mechanism by which *E. faecium* I.3^{rif} is tolerant in CCF, and in *E. faecalis* coculture is unknown. *E. faecium* I.3^{rif} and GRE47 possessed the cell wall adhesion factor *efaAfm*. *E. faecalis* I.2 was positive by polymerase chain reaction for *gelE*, *efaAfs*, *cad*, *ccf*, *cdp*, and *cob*, but not the cytolysin-associated gene *cylMAB*, suggesting that the mechanism of activity against *E. faecium* strains was due to factors other than the two-component cytolysin.

Introduction

One of the most challenging issues in poultry and livestock production is the inability to prevent the dissemination of bacterial pathogens through entire herds or flocks. In many cases bacteria that cause foodborne illness in humans may be commensals in the gastrointestinal tract of animals. Even when the bacteria are pathogenic to poultry and livestock, it is nearly impossible to isolate and quarantine individual animals in the production facilities currently in use in the United States. Antimicrobial treatment of herds and flocks for growth promotion, prophylaxis, or clinical illness is coming under intense scrutiny due to the emergence of antimicrobial-resistant microorganisms in epidemic proportions.

There is great interest in use of probiotics as an alternative for antimicrobials in growth promotion and disease prevention. Efficacious strain selection is dependent on a number of factors, including biosafety, origin of strain, and viability (Kosin and Rakshit, 2006). Viability depends on the ability to withstand antagonistic conditions of the gastrointestinal tract and to colonize the gut of animals that already have a diverse mixture of niche-adapted microflora (Savage, 1977; Brook, 1999). Microorganisms that do not adapt to the local host and/or microbial environment will be eliminated from the niche (Netherwood *et al.*, 1999b). Niche selective pressure may explain why most probiotic preparations fed to adult animals are not maintained after oral administration is discontinued (Netherwood *et al.*, 1999a). Many probiotic microorganisms are selected based on various inhibitory assays and short-term mixed batch cultures (Wagner *et al.*, 2002; Bielke *et al.*, 2003); however, these methods provide no data on the survivability of these isolates in long-term culture, or in a natural niche such as the gut.

Continuous-flow fermentation technology supports bacterial cultures over long periods of time, for example, days, months, or years, as opposed to batch cultures that are limited by the one-time provision of nutrients (Monod, 1949; Pirt,

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1975). This technology can also be applied to mixed bacterial cultures, thus providing a method to study the complex ecology that exists among different bacterial species yet eliminates the host immune response and competition for binding to the intestinal mucosa. In this environment, survival is linked to the ability to compete for nutrients, nutrient affinity and transport, growth rate of different species, and tolerance to volatile fatty acids, bacteriocins, and low pH.

An anaerobic continuous-flow culture of chicken gastrointestinal microflora (CCF) originally established from the contents of a chicken cecum has been previously described (Nisbet *et al.*, 1993; Poole *et al.*, 2001, 2004). CCF contains a diverse mixture of facultative and strict anaerobes, including four species of enterococci, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, and *Enterococcus avium*. Although a strain of *E. faecium* is present in CCF, the culture has been shown to rapidly eliminate exogenous strains of *E. faecium* that have been added to the culture (Poole *et al.*, 2001, 2003, 2004).

During experiments to study the effect of antibiotics on the ecology of CCF, it was concluded that endogenous *E. faecalis* I.2 may play a role in the elimination of exogenous *E. faecium* (Poole *et al.*, 2004). In CCF cultures treated with 10 mg/L of vancomycin (a glycopeptide antibiotic), *E. faecalis* I.2 remained in the culture and exogenous glycopeptide-resistant *E. faecium* (GRE) was eliminated. In cultures treated with 40 mg/L vancomycin, *E. faecalis* I.2 was eliminated and GRE isolate remained in the culture. An agar overlay assay showed that CCF *E. faecalis* I.2 produced a bacteriocin-like substance that possessed inhibitory specificity against *E. faecium*, but not other bacterial or enterococcal species tested (Poole *et al.*, 2001).

Four classes of antibacterial peptides called bacteriocins have been described (Klaenhammer, 1993), and enterococci produce a number of these types of peptides (Franz *et al.*, 2007). Much is known about the *E. faecalis* cytolysin, $CylL_L$ and $CylL_S$, that is a lantibiotic type of enterocin (Gilmore *et al.*, 1990, 1994; Coburn and Gilmore, 2003). However, this two-component peptide is associated with clinical *E. faecalis* isolates rather than environmental *E. faecalis* isolates. The bacteriocin produced by *E. faecalis* I.2 may have a narrower spectrum of activity than has been described for the two-component cytolysin.

One question that arose from previous CCF studies was, how did one strain of *E. faecium* survive in CCF when all exogenous strains exposed to CCF did not? It was hypothesized that this strain adapted to inhibitory factors produced by *E. faecalis* during the initial colonization of the chicken shortly after hatch. The purpose of this study was to further investigate the role *E. faecalis* I.2 may play in toxicity against non-niche-adapted *E. faecium* versus niche-adapted CCF *E. faecium*. It may be possible that selected isolates of *E. faecalis* alone could be used as a competitive exclusion product to reduce carriage of GRE in poultry.

Materials and Methods

Bacterial strains

An anaerobic continuous-flow culture of chicken intestinal microflora (CCF) was established from a defined competitive exclusion culture (CF3). CF3 consists of a mixed culture of indigenous cecal bacteria originally isolated from the cecal contents of a 12-week-old broiler that never received antibiotics. *E. faecium* I.3 was isolated from the CF3 culture, and this strain was made rifampicin-resistant *E. faecium* $I.3^{rif}$. Glycopeptide-resistant *E. faecium* 47 (GRE47), of avian origin from Great Britain, was used as an exogenous *E. faecium* strain and has been previously described (Poole *et al.*, 2004). GRE47 and *E. faecium* $I.3^{rif}$ were isolated from experimental CCF cultures and enumerated on M-Enterococcus medium (Difco, Detroit, MI) with either 32 mg/L vancomycin (Sigma, St. Louis, MO) or 32 mg/L rifampicin (Sigma), respectively. *E. faecalis* I.2 was isolated and enumerated on M-Enterococcus medium with 32 mg/mL erythromycin.

One *E. faecium* strain, P46-32388 (positive for *esp* and *EfaAfm*), and two *E. faecalis* strains, P1-32385 (positive for *gelE*, *cpd*, *cob*, and *EfaAfs*) and P4-32386 (positive for *gelE*, *cylMBA*, *cpd*, *cob*, and *EfaAfs*), were used as controls for polymerase chain reaction detection of potential virulence genes. These strains were generously provided by Dr. Marcus Zervos (Chief, Infectious Diseases Department Internal Medicine, Henry Ford Hospital, Detroit, MI).

Mixed cecal continuous-flow competition and displacement studies

CCF ($n = 1$) was established as a parent culture for replicate experiments, as previously described (Nisbet *et al.*, 1993; Poole *et al.*, 2001, 2004). Each CCF culture for competition experiments was initiated by inoculating a fermentation apparatus containing 1050.0 mL Viande Levure (VL) broth (10.0 g tryptose, 5.0 g yeast extract, 5.0 g NaCl, 2.5 g dextrose, 2.4 g beef extract, and 0.6 g L-cysteine per liter, pH 6.0) with a 100 mL aliquot of the parent CCF culture. The chemostat vessel containing VL broth was flushed with a constant stream of O_2-free CO_2 and was maintained at 39°C. The dilution rate (D) is equal to the flow rate (F) divided by the vessel volume. Each fermentation apparatus had a dilution rate of 0.0417/h, corresponding to a flow rate of 48 mL/h and a vessel turnover time of 24 h. The 24 h turnover time corresponds to the turnover time of the chicken gastrointestinal tract (Nisbet *et al.*, 1993).

The specific growth rate (μ) of the cells in the culture is equal to D if the death rate is negligible. This is theoretically the case in continuous-flow systems where exponential growth is maintained. The washout rate is a calculated value

$$\ln X = (\mu - D)t + \ln X_o$$

when the specific growth rate $\mu = 0$.

Each CCF culture was maintained for 1 week before initiation of an experiment. A successfully stabilized culture was determined by a stable pH of 5.8–6.0 and a concentration of 5.0–6.0 $\log_{10}$ cfu/mL *E. faecalis*. Each competition study ($n = 3$) was initiated by inoculating the CCF culture with 11.5 mL each of an overnight pure culture of GRE47, or *E. faecium* $I.3^{rif}$. One milliliter aliquots were serial diluted for enumeration on M-Enterococcus medium with antibiotics (as described above) and were taken daily from all replicate experimental cultures. The lower cfu/mL detection limit was 10^{-2}.

For displacement studies ($n = 3$) 11.5 mL of a pure overnight culture of GRE47 was inoculated into a fermentation apparatus containing 1138.5 mL VL broth. The GRE47 monoculture was maintained for 3 days without pH adjustment. At 72 h postinoculation, a 100 mL aliquot of the parent

CCF culture was added to the GRE47 monoculture. Aliquots for serial dilution and enumeration on M-Enterococcus medium with rifampicin 32 mg/L were taken daily from all replicate experimental cultures.

Continuous-flow coculture competition studies

Each experimental continuous-flow coculture ($n = 3$) was initiated by inoculating a fermentation apparatus containing 1127.0 mL VL broth with 11.5 mL each of an overnight pure culture of *E. faecalis* I.2 and GRE47, or *E. faecalis* I.2 and *E. faecium* I.3^{rif} to a total volume of 1150.0 mL. The flow rates and dilution rates were as described. Aliquots for enumeration were taken daily from all replicate experimental cultures.

Antibiotic rescue

If an isolate was reduced below detection limits in an experimental culture, the culture was treated with 100 mg/L of the appropriate selective antimicrobial 3 days after the last time point the isolate was detected. The culture was monitored by plating on M-Enterococcus medium with the same antimicrobial used to determine if the isolate could rebound under selective pressure. If the isolated could not be cultured under strong selective pressure, it was considered eliminated from the culture.

Detection of potential virulence factors

Polymerase chain reaction to detect putative virulence factors with the potential to inhibit exogenous *E. faecuim* was done for the following genes: *agg*, *gelE*, *cylMBA*, *esp*, *efaAfs*, *efaAfm*, *cpd*, *cob*, and *ccf*. Total DNA for *E. faecalis* I.2 *E. faecium* I.3^{rif}, and GRE47 was tested as previously described (Eaton and Gasson, 2001).

Statistical analysis

Regression analysis was used to find the *y*-intercept and compare slopes of estimated and observed dilution curves using Statistix 9 analytical Software (Tallahassee, FL).

Results

Survivability of E. faecium *I.3^{rif} or GRE47 in chicken continuous-flow cultures*

Upon inoculation of CCF cultures ($n = 3$) with GRE47, the mean GRE47 population was 6.4 $\log_{10}$ cfu/mL $\pm$ 0.5 (Fig. 1). Subsequently, GRE47 was eliminated from all replicate CCF cultures at a rate of 1.01 $\log_{10}$ cfu/mL/day and was below detection limits by day 7. A comparison of regression lines obtained from the decline in GRE47 population to the calculated washout rate was statistically different ($p < 0.05$). The addition of 100 mg/mL vancomycin on day 10 did not result in a rebound of GRE47 in the CCF culture.

In separate CCF cultures ($n = 3$), the initial mean population of *E. faecium* I.3^{rif} was 5.8 $\log_{10}$ cfu/mL $\pm$ 0.1 (Fig. 1). In the next 48 h *E. faecium* I.3^{rif} declined by 2.2 $\log_{10}$ cfu/mL, but the populations returned to approximately 6.0 $\log_{10}$ cfu/mL by 72 h postinoculation. The abrupt 100-fold decrease followed by a return to a stable population was observed in all three replicate cultures. The *E. faecium* I.3^{rif} population peaked on day 5 at 6.7 $\log_{10}$ cfu/mL $\pm$ 0.2. There was a slight decline in the *E. faecium* I.3^{rif} population throughout the remainder of the experiment with a final concentration on day 21 of 5.3 $\log_{10}$ cfu/mL $\pm$ 0.6. The 2 log decrease may represent the elimination of less-fit *E. faecium* I.3^{rif} cells. By performing linear regression on the data points during the rebound phase and extrapolating back to the zero time point, the initial fit *E. faecium* I.3^{rif} population was calculated at 3.1 $\log_{10}$ cfu/mL. This represented 0.17% of the initial *E. faecium* I.3^{rif} population.

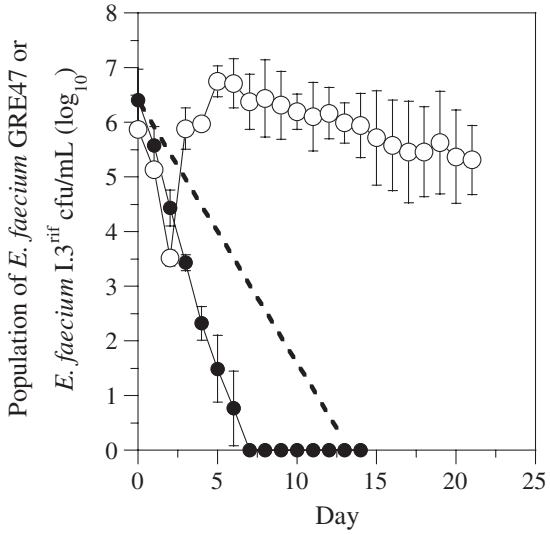

FIG. 1. *Enterococcus faecium* I.3^{rif} (I.3^{rif}) or glycopeptide-resistant *E. faecium* 47 (GRE47) populations in separate anaerobic continuous-flow culture of chicken gastrointestinal microflora (CCF) cultures (values are mean $\pm$ SD; $n = 3$). (●) GRE47 population; (○) *E. faecium* I.3^{rif} population; (—) hypothetical level if disappearance was only due to washout. The data for separate GRE47 and I.3^{rif} are plotted on one graph.

Displacement of GRE47 by CCF

Continuous-flow monocultures ($n = 3$) of GRE47 were established to determine if GRE47 could remain or adapt to CCF if GRE47 occupied the culture vessel to full capacity. On day 3 postinoculation, before exposure to CCF, the mean population of GRE47 was 8.9 $\log_{10}$ cfu/mL (Fig. 2). Approximately 72 h postinoculation a 100 mL aliquot of CCF was added to the GRE47 monocultures. Despite the relatively small inoculum of CCF, GRE47 was displaced from the culture at a rate of 0.6 $\log_{10}$ cfu/mL/day. By day 17 GRE47 populations were below detection limits in all replicate cultures. On day 21 postinoculation, 100 mg/mL vancomycin was added to the culture; however, antibiotic treatment failed to result in a rebound of GRE47 in any of the cultures.

Survivability of E. faecium *I.3^{rif} or GRE47 in coculture with* E. faecalis *I.2*

In continuous-flow cocultures of *E. faecalis* I.2 and GRE47 ($n = 3$) that were both inoculated on the same day, the GRE47 population increased for the first 2 days then decreased at a rate of 1.2 $\log_{10}$ cfu/mL/day until day 10 when it was below detection limits (Fig. 3A). A comparison of regression lines obtained from the decline in GRE47 population to the calculated washout rate was statistically different ($p < 0.0001$). On

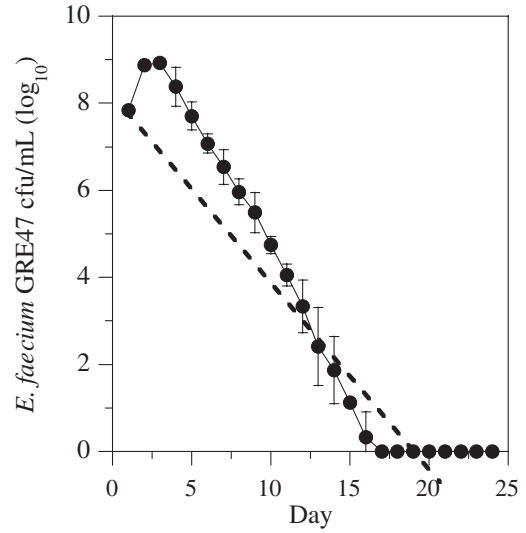

FIG. 2. GRE47 population displacement by CCF cultures (values are mean ± SD; $n = 3$). (●) GRE47 population; (—) hypothetical level if disappearance was only due to washout.

day 15 postinoculation, 100 mg/L vancomycin was added to all replicate cocultures. GRE47 was not detected on M-Enterococcus medium with 32 mg/L vancomycin after day 15. Additionally, by day 18 no enterococci were detected on M-Enterococcus agar either with antimicrobials or without 32 mg/L at a concentration of erythromycin (data not shown), suggesting that the vancomycin killed all *E. faecalis* I.2.

In continuous-flow cocultures of *E. faecalis* I.2 and *E. faecium* I.3^{rif} ($n = 3$), the *E. faecium* I.3^{rif} population was present at 7.5–7.0 $\log_{10}$ cfu/mL for first 4 days, and then demonstrated a moderate decline for 7 days followed by an increase in populations back to 7.8 $\log_{10}$ cfu/mL by day 21 (Fig. 3B). The decline in the *E. faecium* I.3^{rif} population may have represented the elimination of cells that could not coexist in the presence of *E. faecalis* I.2. As in the CCF culture, the repopulating *E. faecium* I.3^{rif} may represent an expansion of a tolerant clone. The initial decline in *E. faecium* I.3^{rif} occurred after a 2 log increase in the *E. faecalis* I.2 population. Linear regression of the tolerant *E. faecium* I.3^{rif} population suggests that the initial population was approximately 3.9 $\log_{10}$ cfu/mL. This represented 0.02% of the initial *E. faecium* I.3^{rif} population.

Detection of potential virulence factors

The sex pheromone–encoding genes (*ccf*, *cob*, and *cpd*) were present in the positive control strain and *E. faecalis* I.2, but not in *E. faecium* I.3^{rif} or in GRE47. *E. faecalis* I.2, *E. faecium* I.3^{rif}, and GRE47 were all negative for *cylM*, *cylB*, or *cylA*. *E. faecium* I.3^{rif} and GRE47 were positive for only *efaAfm*.

Discussion

Because four enterococcal species (*E. faecalis* I.2, *E. faecium* I.3, *E. gallinarum* I.5, and *E. avium* I.6) coexisted in CCF, it was originally thought that CCF would easily support other *E. faecium* strains. However, previous studies have shown that *E. faecium* species foreign to CCF could not survive in the

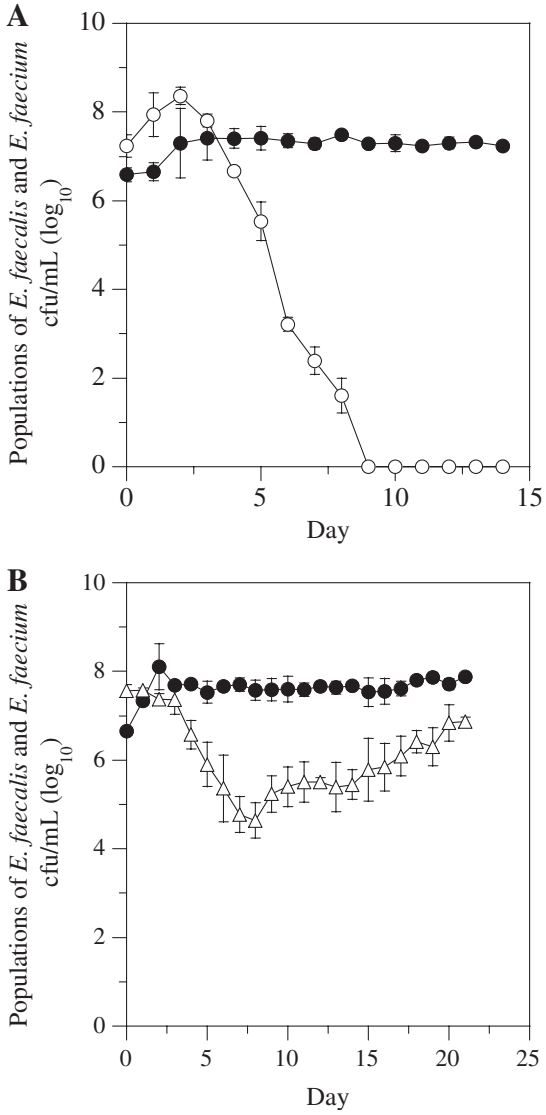

FIG. 3. *E. faecium* I.3^{rif} (I.3^{rif}) and GRE47 populations in coculture with *Enterococcus faecalis* I.2 (values are mean ± SD; $n = 3$). (**A**) ●, *E. faecalis* I.2 population; ○, GRE47 population. (**B**) ●, *E. faecalis* I.2 population; Δ, *E. faecium* I.3^{rif}.

culture (Poole *et al.*, 2001, 2004). A bacteriocin overlay assay using *E. faecalis* I.2 as the bacteriocin-producing strain indicated inhibitory activity with specificity against six *E. faecium* isolates, but not other bacterial species *E. gallinarum*, *E. avium*, *E. faecalis*, *Lactobacillus* sp., *Lactobacillus sakei*, *Lactococcus* sp., or *Streptococcus bovis* (Poole *et al.*, 2001). Although *E. faecium* I.3 was tolerant of the inhibitory conditions in CCF, it was inhibited by *E. faecalis* I.2 in the bacteriocin assay. The *E. faecium* I.3 isolate that was used in the bacteriocin assay had been cultured and stored as freezer stocks and may have lost its adaptive phenotype. Or the difference in physical proximity between the bacteria in the bacteriocin assay, on an agar plate, versus a broth continuous-flow culture may have played a role in the inhibition of *E. faecium* I.3.

In both CCF and *E. faecalis* I.2 cocultures with *E. faecium* I.3^{rif}, it appeared that only a small subset of the *E. faecium* I.3^{rif}

was tolerant to the inhibitory conditions. The tolerant population of *E. faecium* I.3^{rif} in CCF was 3.1 $\log_{10}$ cfu/mL or 0.17% of the initial *E. faecium* I.3^{rif} population. The *E. faecium* I.3^{rif} population may have become susceptible to the inhibitory factors of CCF because it had been isolated and grown *in vitro* to select for rifampicin-resistant clones. In the *E. faecalis* I.2 and *E. faecium* I.3^{rif} cocultures, 3.9 $\log_{10}$ or 0.02% of the *E. faecium* I.3^{rif} population was tolerant of inhibitory conditions. However, in this culture the rate of initial elimination of the susceptible population was not as rapid. It is likely that multiple inhibitory factors are produced by other bacterial species in CCF compared to the coculture environment and that may have caused a more rapid inhibitory effect. Since the population of tolerant *E. faecium* I.3^{rif} in the *E. faecalis* I.2 coculture and in CCF was within the same log, this may suggest that most of the inhibitory specificity observed by CCF against *E. faecium* spp. may be due to cytotoxic factors expressed by *E. faecalis* I.2.

GRE47 was eliminated from CCF at a rate of 1.01 $\log_{10}$ cfu/mL/day. This rate was faster than the washout rate, which may suggest that CCF exhibits bactericidal activity against GRE47. If CCF activity was bacteriostatic and arrested growth of GRE47, the expected removal rate would have approximated the calculated washout rate. GRE47 was eliminated from a coculture with *E. faecalis* I.2 at a slightly slower rate than from CCF; however, these differences may be due to differences in initial populations or ecological differences between a complex mixed culture and the enterococcal cocultures. In an attempt to determine if GRE47 could adapt to CCF if it initially existed as a monoculture, a displacement study was done. Under the conditions used in this study, GRE47 was unable to adapt to CCF. GRE47 could not be rescued with vancomycin in any of the competition studies, suggesting that it had been killed, not growth arrested. No inhibitory effect was observed on *E. faecalis* I.2 from either of the *E. faecium* strains in coculture. This is consistent with the observation that *E. faecalis* I.2 maintains the highest enterococcal population in CCF.

A number of virulence factors have been described for *E. faecalis*, including proteins for aggregation, cell wall adhesions, sex pheromones, and those related to the two-component cytolysin (Mundy *et al.*, 2000). The cytolysin produced by *E. faecalis* has been well characterized (Coburn and Gilmore, 2003); however, very little is known about how it may affect other bacteria in a complex microbial consortium. It has been suggested that cytotoxin evolution may have occurred as a necessity for control in a complex ecological niche such as a soil habitat (Coburn and Gilmore, 2003). However, in this study *E. faecalis* I.2 did not possess the cytolysin-associated gene *cylMAB*, suggesting that the mechanism for inhibition of *E. faecium* I.3^{rif} was due to inhibitory factors other than the two-component cytolysin. It was thought that if *E. faecalis* I.2 had possessed the cytolysin operon, then *E. faecium* I.3^{rif} may have acquired the cytolysin-associated immunity gene. However, *E. faecium* I.3^{rif} was also negative for cytolysin-related genes.

It is speculated that *E. faecium* I.3 sequentially coadapted to multiple inhibitory factors in the chicken caecum during the initial microbial colonization, thus enabling survival. Once the cecal niche matured, it may have become more difficult for an exogenous species to adapt to the cocktail of inhibitors present before they were eliminated.

The data from this study show that nonadapted bacterial strains have reduced survivability in stable complex bacterial mixtures.

Acknowledgments

We thank Reiley Street for technical assistance.

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Disclosure Statement

No competing financial interests exist.

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